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PHOTOREACTIONS OF MELANIN: A NEW TRANSIENT SPECIES AND EVIDENCE FOR TRIPLET STATE INVOLVEMENT

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Summary. The detection of a new transient species in photoirradiated natural and synthetic melanins is reported. This species decays rapidly at both ambient and cryogenic temperatures and has an electron spin resonance spectrum whose time-profile reveals spin polarization effects (chemically induced dynamic electron polarization) characteristic of triplet state involvement. A possible mechanism for light absorption and degradation by melanins is suggested.

Introduction. The major biological function of melanin pigments is believed to be photoprotection, yet the mechanism of their interaction with light is poorly understood. Melanins are redox polymers, containing high concentrations of o-quinone and o-hydroquinone groups.[1,2] They also possess paramagnetism[3] (thought to be due to o-semiquinone free radicals) which has been the subject of several electron spin resonance (ESR) investigations.[4] While paramagnetic species are always present in neutral melanin suspensions (intrinsic free radicals†) they also can be induced by various chemical and physical treatments, including irradiation with visible or ultraviolet light.[5,6]

The light-induced radicals have been reported to be transient at room temperature, with a first half-life of ca. 1 s,[6] and to be stabilized at cryogenic temperatures.[5] The room temperature decay has been fitted[6] to a complex kinetic model. However, the time-resolution available in these experiments was only 0.1 s. Using a pulse-photolysis system we have now made

†To be consistent with other reports,[5,6] we shall also loosely describe the paramagnetic species as "free radicals", although[4] they should not necessarily be regarded as isolated S=1/2 species.

Abbreviations: ESR - Electron Spin Resonance, Dopa - β-dihydroxylphenyl alanine, CIDEP - Chemically Induced Dynamic Electron Polarization.

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ESR measurements on irradiated natural and synthetic melanin preparations with time resolution down to 0.2 ms. The data obtained reveal the presence of a much faster decaying spectral component that we believe is derived from a triplet state. It has also been possible to show that the light-induced radicals are spectroscopically distinct from the intrinsic ones.

Methods. Samples of bovine eye melanin and synthetic melanin from DL-β-dihydroxyphenylalanine (Dopa) were prepared as described by Plumer and Kopac[7] and by Felix et al.[8] respectively. Synthetic melanins were dialyzed before use. Whereas the synthetic material is protein- and metal-free[9], the natural material isolated in this manner contains both endogeneous protein[10] and metal ions. These largely can be removed by treatment with concentrated (6M) hydrochloric acid.[11]

All samples were used in the form of aqueous suspensions at pH 7. Drying of aliquots showed that they contained ca. 10 mg/ml of melanin. The suspensions were rigorously deoxygenated before measurements were made, either by freeze-pump-thaw cycles on a vacuum line or by a short period of photolysis.[12] Where comparisons were made, the two methods gave identical results.

Samples were irradiated at temperatures between -196 and +80 °C in situ in the cavity of a Varian E-109 spectrometer with filtered light from an Eimac VIX-300UV 300 W xenon lamp. Irradiation at 320 - 600 nm was generally employed; a filter of 5 cm of $CuSO_4$ solution (100 g l^{-1}) was used for this purpose. In other experiments colored glass filters were used to select wavelength regions for irradiation. Time-resolved ESR spectra were obtained by setting the magnetic field on the maximum of the ESR first derivative and, depending upon the time resolution required, modulating the light intensity electronically using a system similar to that described by Levanon and Weissman[13] or by means of a mechanical shutter assembly.

Results and Discussion. It has not been clear from previous ESR measurements how (if at all) the intrinsic and light-induced radicals differ. We have obtained steady-state spectra for light-induced radicals by subtracting the intrinsic (i.e. dark) spectrum from the composite spectrum observed during continuous photolysis. ESR parameters for intrinsic and light-induced species are given in the Table. It is apparent that the spectra differ in both line-shape and g-value and in their microwave saturation characteristics. It was established by selective filtering that free radicals are generated by light throughout the visible and near ultraviolet spectrum.

Time-resolved experiments at long observation times (Fig. 1(a)) show, in agreement with earlier reports,[6] a slowly decaying transient species in suspensions of photoirradiated bovine eye melanin at 25 °C. In our hands the slow decay follows excellent second order kinetics (see Fig. 1 (b)) with a

Table. ESR Parameters for Intrinsic and Light-Induced Free Radicals in Natural and Synthetic Melanins at 25 °C.

Melanin	g[a]	linewidth/G[b]	$P_{1/2}$/mW[c]
Bovine eye:			
intrinsic radicals	2.0041	4.7	7 ± 2
light-induced radicals	2.0045	5.4	14 ± 2.4
Dopa:			
intrinsic radicals	2.0034	3.8	2.5 ± 0.1
light-induced radicals	2.0039	3.9	11 ± 1.3

[a] ± 0.0001; [b] ± 0.1 G; [c] Data for deoxygenated aqueous suspensions. $P_{1/2}$ is the microwave power at which the ESR signal is half as large as it would be in the absence of saturation.

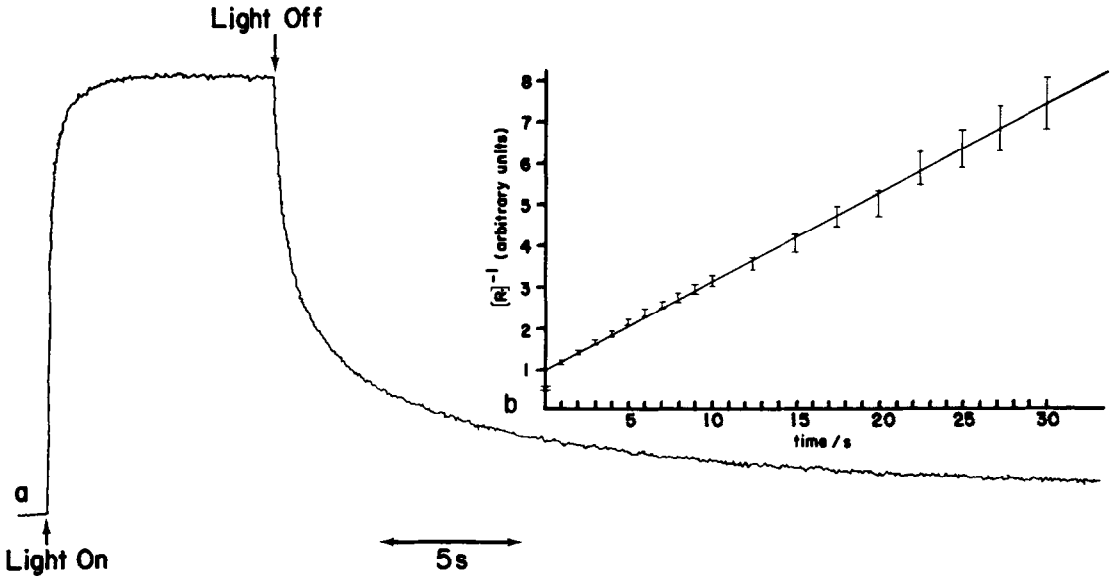

Fig. 1(a). Time-resolved ESR spectrum of photo-induced free radicals in natural melanin from bovine eyes, obtained by mechanical modulation of the light intensity. Temperature = 25 °C. Signal average of 28 scans; signal averager filter time 40 ms. (b) Second-order plot of the data; [R·] = transient free radical concentration.

first half-life of 5 s for an initial transient radical concentration of ca. 1 x 10^{-5} M. However, a contribution from a fast-decaying component is also apparent.

At shorter times, this fast component dominates the radical decay (Fig. 2 (a)). Although we have not yet been able completely to separate the two

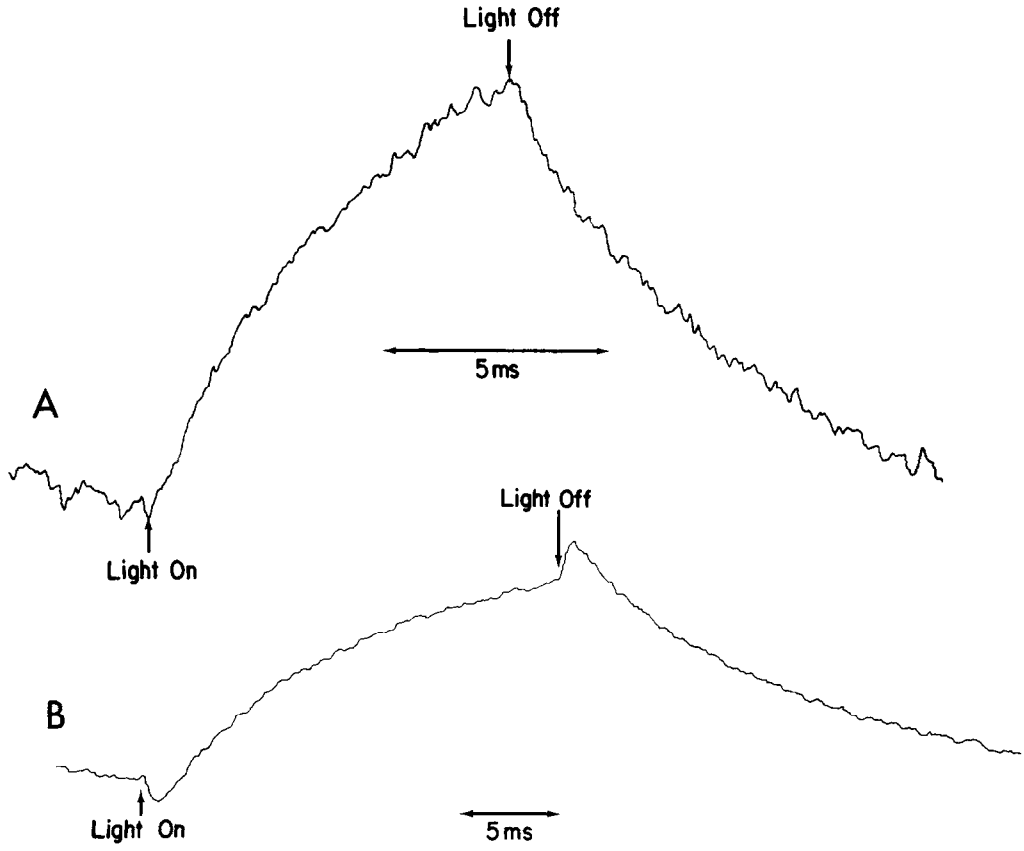

Fig. 2. Time-resolved ESR spectra of transient free radicals, obtained by electronic modulation of the incident light intensity. Temperature = 25 °C. (a) Natural melanin from bovine eyes. Signal average of 5,500 scans. (b) Synthetic dopa-melanin. Signal average of 16,384 scans. In these displays radical concentrations do not decay to zero during the light off period.

decays, variable temperature measurements indicate a large temperature dependence for the slow component, little or no temperature dependence for the fast one. Thus, a profile similar to that shown in Fig. 2(a) was also obtained at -196 °C. The two components contribute approximately equally to the spectrum whose parameters are reported in the Table. However, the yield of the fast component is very much (50 - 100 times) greater than that of the slow component, based on its contribution to the steady state signal obtained during continuous photolysis and its approximate chemical lifetime (50 ms).

Synthetic melanin preparations also display slow and fast kinetic components. However, in this instance chemically induced dynamic electron

polarization (CIDEP) effects are evident at short observation times (Fig. 2(b)). This kind of time profile has been encountered in chemical systems,[14] where it is characteristic[15] of electron spin polarization induced by the triplet mechanism.[16] In the familiar form of the latter, light absorption by species A leads to its promotion to an excited singlet state A^S, which intersystem crosses to the corresponding triplet, A^T. Intersystem crossing to each of the triplet sublevels is not equally favored, and an imbalance of the population of the sublevels (i.e. spin polarization) results. Provided that reaction of the triplet, e.g. with hydrogen-atom donor DH, is fast enough to compete with its loss of polarization via spin-lattice relaxation the polarization can be transferred to radical products, as in reaction (1) where * indicates a spin-polarized species. Polarization in free radicals is eventually also lost through spin-lattice relaxation.

$$A \longrightarrow A^S \longrightarrow (A^T)^* \longrightarrow (AH^\cdot)^* + (D^\cdot)^* \qquad (1)$$

We therefore infer that the transient free radicals in synthetic melanin have a triplet state precursor. We have obtained similar spectra from <u>natural</u> melanin but only after treatment with acid, which serves[17] to reduce the concentration of bound metal ions. It seems that paramagnetic ions such as Cu^{2+} and Fe^{3+}, which are commonly found in bovine eye melanin,[10] are able to suppress spin polarization by promoting relaxation in the triplet and free-radical states. Thus, the addition of Cu^{2+} to synthetic melanin in the ratio of 1 Cu^{2+}:200 melanin monomers was found to completely quench CIDEP whereas a similar concentration of Zn^{2+} (diamagnetic) was without effect.

Given the facility of photoreaction between quinones and hydroquinones,[18] and the preponderance of such groups on the melanin polymer, we suggest that light absorption by quinone groups or quinhydrone complexes[8] leads to chemical reaction as in (2), where the pair of semiquinone radicals formed undergoes recombination, to leave the structure of the polymer unchanged. (We have yet to detect a change in the free-radical properties of deoxygenated melanins, even after many hours of continuous photolysis). It seems likely that a cyclic

mechanism of this kind plays a part in the photoprotective action of the polymer.

$$Q + QH_2 \xrightarrow{h\nu} \text{singlet} \longrightarrow \text{triplet} \longrightarrow QH^{\cdot} + QH^{\cdot} \longrightarrow Q + QH_2 \quad (2)$$

where Q, QH_2, and $QH^{\cdot}$ represent quinone, hydroquinone and semiquinone units on the polymer, respectively. Possible ionizations of the units are not shown.

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